Chronic Antioxidant Supplementation Impairs Coronary Endothelial Function and Myocardial Perfusion in Normal Pigs

Daniele Versari, Elena Daghini, Martin Rodriguez-Porcel, Katherine Sattler, Offer Galili, Kevin Pilarczyk, Claudio Napoli, Lilach O. Lerman, Amir Lerman

Abstract—Experimental studies have shown the beneficial effects of antioxidant supplementation on endothelial function in the presence of increased endogenous oxidative stress, whereas limited data are available under normal conditions. The present study tested the hypothesis that in normal pigs long-term antioxidants would have deleterious effects on the cardiovascular system. Normal domestic pigs (V, n=6) were studied 12 weeks after dietary supplementation with vitamin E (100 IU/kg per day) and vitamin C (1 g/day) and compared with normal controls (C, n=7). Myocardial perfusion and permeability index were evaluated by electron beam computed tomography after intravenous adenosine and dobutamine. Coronary endothelial function was evaluated in vitro by organ chamber and coronary tissue studied by immunoblotting and staining. Myocardial perfusion response was lower in V than in C after adenosine (10.1±4.5 versus 53.4±5.2%; P<0.01) and dobutamine (V, 78.4±8.1; C, 193.0±39.0%; P<0.05). The permeability index increased in V after adenosine (48.8±5.1%) and dobutamine (59.9±13.6%) and did not change in C. Coronary vasodilation to bradykinin and substance P was lower in V than in C. Moreover, V coronary nitrotyrosine and superoxide content was significantly higher than in C. The groups had similar total monomer expression of endothelial nitric oxide synthase, whereas the dimerized form, reflecting coupled enzyme, was lower in V. These findings suggest that long-term experimental antioxidant vitamin supplementation in normal pigs impairs myocardial perfusion and coronary endothelial function via an increased level of oxidative stress in the arterial wall, which may be partly related to the uncoupling of endothelial nitric oxide synthase and/or the direct prooxidant effect of vitamin radicals. (Hypertension. 2006;47:475-481.)

Key Words: antioxidants ■ endothelium ■ myocardial ■ oxidative stress ■ vasodilation ■ nitric oxide synthase
tein (LDL) oxidizability,14 and prevent renal cortical15 and myocardial16 neovascularization. We also showed that long-term antioxidant vitamin supplementation is able to improve the impaired myocardial perfusion and permeability responses to increased cardiac demand in a model of porcine hypertension.17 On the other hand, in the presence of low oxidative stress, antioxidant vitamins may accumulate and paradoxically produce prooxidant species. However, the effect of similar antioxidant vitamin supplementation in animals under normal conditions has not been established. Thus, the present study was designed, using the porcine experimental model as an integrate approach system, to test the hypothesis that chronic antioxidant vitamin supplementation in normal animals might result in detrimental effects on myocardial perfusion and permeability, as well as on coronary endothelial function and systemic and vascular oxidative stress.

Methods

Animals
All of the study procedures were approved by Mayo Foundation Institutional Animal Care and Use Committee. Thirteen female juvenile domestic crossbred pigs (55 to 65 kg) were randomized into 2 groups. The first group (C: n=7) received a normal pig chow diet (Land O’ Lakes Purina Feed), containing small amounts of essential vitamins and minerals; the second group (V; n=6) was fed the same normal diet but was additionally supplemented with a combination of antioxidant vitamin C and vitamin E (ascorbic acid 1 g/day and dl-α-tocopherol acetate 100 IU/kg per day), which we proved previously to be effective in decreasing oxidative stress and normalizing endothelial function and myocardial perfusion in pig models of hypertension and/or hypercholesterolemia.14,15,17,18 After 12 weeks, blood samples were collected for the measurement of plasma lipid profile and parameters of oxidative stress. Electron beam computed tomography (Imatron C-150, Imatron Inc) studies were performed to assess myocardial perfusion and microvascular permeability.17–19 The animals were then euthanized (Sleepaway, 10 mL; Ft Dodge Laboratories), the hearts immediately harvested, and coronary arteries dissected, placed in control solution, and sectioned into rings to study endothelial function.

Myocardial Perfusion and Permeability Studies
Myocardial perfusion and permeability were assessed by electron beam computed tomography, as described previously.18,19 Briefly, pigs were anesthetized with an intramuscular bolus of ketamine (30 mg/kg) and xylazine (5 mg/kg), and anesthesia was maintained with a constant infusion of ketamine 0.3 mg/kg per minute and xylazine 0.04 mg/kg per minute). After stabilization, 40 consecutive ECG-triggered end-diastolic scans were obtained at the middle-left ventricle level, after injecting a bolus contrast agent iopamidol (Isovue-370, Squibb Diagnostics). Thirty minutes after baseline, functional studies were repeated after 10- to 15-minute IV infusion of adenosine (400 μg/kg per minute) and dobutamine (15 μg/kg per minute, target heart rate of 150 bpm).

Data Analysis
Detailed procedures have been described previously.18,19 Briefly, regions of interest were traced within the anterior wall and chamber of the left ventricle (Image analysis software Analyze, Mayo Foundation), and time-density curves were obtained. Curves were fitted with an extended γ-variate algorithm (Kaleidagraph, Synergy Software) to model curves representing the transit of contrast in the intravascular and the extravascular compartment.18–20 Myocardial perfusion (mL/g per minute) and microvascular permeability index [arbitrary units (AU)] were calculated according to previously validated formulas.18–20

In Vitro Analysis of Vascular Reactivity
In vitro coronary endothelial function was assessed by the organ chamber technique.21,22 Briefly, after precontraction, coronary rings were challenged with the endothelium-dependent vasodilators bradykinin (10−10 to 10−6 mol/L, Sigma) and substance P (10−8 mol/L, Sigma) and with the endothelium-independent stimulus papaverine (10−5 to 10−3 mol/L, Sigma); vessel wall tension was recorded by an isometric force transducer.21,22 The ED50 was calculated by nonlinear regression analysis, fitting concentration-response plot.

Oxidative Stress Evaluation
Plasma Parameters of Oxidation and Tissue Superoxide Dismutase
LDL oxidizability, as Lag time,23 malondialdehyde,23 relative electrophoretic mobility of lipoproteins,23 thiobarbituric acid–reactive substances (TBARS),24 and plasma vitamin E and C18 were evaluated. Superoxide dismutase (CuZn- and Mn-SOD) activity was determined spectrophotometrically, as described previously,18 and normalized for protein content.

Immunohistochemistry for Nitrotyrosine
After deparaffinizing and hydrating, slides were incubated overnight at 4°C with primary antibody (rabbit polyclonal; Sigma; 1:1000) and then for 1 hour with anti-rabbit IgG secondary antibody (Dako Laboratories, Glostrup, Denmark). Diaminobenzidine was used as chromogen. Slides were viewed under microscope (Olympus, Leeds Precision Instruments) and analyzed with imaging software (MetaMorph, Meta Imaging series 4.6). On hematoxylin and eosin slides, the region of interest (intima) was defined internally to the internal elastic lamina and transferred to the nitrotyrosine-stained slides. The percentage of stained intima was semiautomatically quantified.

In Situ Detection of Superoxide Anion
In 30-μm frozen coronary sections, the in situ production of superoxide anion was measured using the oxidative fluorescent dye dihydroethidium (DHE), as described previously.25,26 An imaging program (MetaMorph) was used to calculate the ratio between red and blue fluorescence, the higher ratio being an index of higher superoxide content.

Immunoblotting for Endothelial NO Synthase and Endothelial NO Synthase Nitration
Freshly frozen coronary artery were homogenized27 and protein content analyzed by Bradford assay (Bio-Rad). Equal amounts of protein were resolved in two 8% sodium dodecyl sulfate polyacrylamide gels. Parallel experiments were performed under reducing conditions and by boiling the samples [to assess the total endothelial NO synthase (eNOS) monomer expression] and under nonreducing condition and without boiling the samples (to assess the dimer content, as described by Ravi et al28). Immunoblotting was performed using monoclonal antibodies for eNOS (Transduction Laboratories, Lexington, KY; 1:200) and secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham Life Sciences; 1:1000); membranes were developed with chemiluminescence (Pierce)29 and exposed to X-ray film (Kodak). Signals were evaluated for integrated density using ImageJ (National Institutes of Health). β-Actin (Sigma; 1:1000) was used as the loading control.

To evaluate eNOS nitration, we performed immunoprecipitation for eNOS followed by a Western blot analysis for nitrotyrosine. Equal amounts of protein were incubated with monoclonal anti-eNOS antibody (Transduction Laboratories) overnight at 4°C with gentle shaking. Protein A/G PLUS-agarose (Santa Cruz) was added and incubated for 2 hours. The beads were washed repeatedly with lysis buffer, and immune complexes were resolved in 8% sodium dodecyl sulfate polyacrylamide gels. Immunoblotting was performed using rabbit anti-nitrotyrosine (Sigma, 1:5000) and secondary anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham, 1:100 000). Membranes were developed and signals analyzed as described above.
Statistical Analysis
Results are presented as mean±SEM. Within each group, repeated measurements were analyzed with repeated measures ANOVA followed by the Bonferroni t test, or by unpaired Student’s t test between groups. A P value <0.05 was used to define statistical significance.

Results
At the end of the diet period, the 2 groups of pigs had similar body weight, lipid profile, and mean arterial pressure (Table). There was no difference between the groups in the systemic hemodynamic response to adenosine or dobutamine (Table).

Myocardial Perfusion
Baseline myocardial perfusion of the left ventricular anterior wall was similar between the 2 groups (C, 0.89±0.10; V, 0.95±0.11 mL/min per gram of tissue). After adenosine, myocardial perfusion significantly increased in C (to 1.31±0.14 mL/min per gram of tissue; P<0.05 versus baseline) but remained unchanged in V (to 0.99±0.20 mL/min per gram of tissue). The percentage of response to adenosine was significantly greater in C (53.4±5.2 versus 10.1±4.5%; P<0.001; Figure 1). Intravenous dobutamine caused a significant increase in myocardial perfusion from baseline in both C and V (2.29±0.30 and 1.84±0.21 mL/min per gram of tissue, respectively; P<0.001 for both groups), but the percentage increase of myocardial perfusion was significantly greater in controls (193.0±39.0 versus 78.4±8.1%; P<0.05; Figure 1).

Myocardial Microvascular Permeability
Baseline permeability index, a parameter of microvascular function in vivo, was similar in the 2 groups (C, 1.50±0.13; V, 1.49±0.19 AU). The permeability index increased significantly in V after infusion of both adenosine (2.45±0.19 AU; P<0.05; 48.8±5.1%), and dobutamine (2.10±0.23 AU; P<0.01; 59.9±13.6%). No significant changes were seen in C in response to either adenosine (1.73±0.19 AU; 11.2±4.6%) or dobutamine (1.87±0.25 AU; 14.8±7.3%) (Figure 1).

Coronary Endothelial Function
The 2 groups had similar precontraction of epicardial vessels to endothelin 1. Vasorelaxation to substance P was significantly attenuated in V as compared with C (maximal vasorelaxation: 47.8±11.8% versus 91.3±3.3%, respectively; P<0.01; Figure 2), with no difference in the ED50 between the 2 groups (C, −8.08±1.33; V, −8.0±0.58). Moreover, V showed a reduced vasorelaxation to bradykinin (area under the curve: 576±26 versus 752±27 AU; P<0.001; maximal vasorelaxation: 62.0±6.5 versus 95.4±1.6%; P<0.01; Figure 2), with a significant right-shift of the ED50 (C, −7.76±0.2; V, −8.56±0.52; P<0.01). Vasorelaxation to the endothelium-independent stimulus papaverine was similar between the 2 groups.

Evaluation of Oxidative Stress and eNOS Expression
At the end of the follow-up, plasma vitamin C and vitamin E levels were significantly higher in V than C (Table 1); plasma LDL oxidation and oxidizability were significantly reduced in the V group, with longer lag phase and lower relative electrophoretic mobility of lipoproteins and malondialdehyde than in C; plasma TBARS remained unchanged (Table 1). Coronary Cu/Zn-SOD activity was similar in the 2 groups (C, 8.6±0.4; V, 9.5±0.7 IU/mg protein), whereas Mn-SOD activity was increased in V (C, 2.6±0.1; V, 3.1±0.1 IU/mg protein).

LDL-REM indicates relative electrophoretic mobility of lipoproteins; MDA, malondialdehyde; HR, heart rate; MAP, mean arterial pressure. *P<0.01, †P<0.001.
Moreover, V showed a significant positive immunostaining for nitrotyrosine in the intima of coronary arteries. The intimal area positive for the staining was significantly higher in V (Figure 3). DHE fluorescence also showed a significant increase in local superoxide production in V (Figure 4). Western blotting analysis of eNOS indicated no significant difference in the expression of the monomeric enzyme between the 2 groups, suggesting a similar overall expression of eNOS protein. On the contrary, a statistically significant decrease in the dimeric form was seen in V as compared with C (Figure 5a), suggesting the uncoupling of eNOS in vitamin-supplemented pigs. Immunoblotting for nitrotyrosine performed after eNOS immunoprecipitation showed a significantly increased nitration of eNOS in V (Figure 5b).

Discussion

The current study demonstrates that chronic administration of high-dose antioxidant vitamins in normal pigs, which are characterized by a low endogenous vascular oxidative stress, results in a significant impairment in myocardial perfusion and coronary endothelial function. Along with the functional alteration, an increased endogenous vascular wall oxidative stress was observed. These data suggest that antioxidant vitamins may have a detrimental effect on the cardiovascular system in healthy subjects through the imbalance of the endogenous redox equilibrium.

Oxidative stress can be defined as an "imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage." This definition implies a balance between prooxidants and antioxidants and led to the concept of "reductive stress" to describe a condition where the balance is altered in favor of reductants. The overproduction of reducing equivalents can eventually cause an increased generation of superoxide anions. Although vitamin E and vitamin C are
generally considered antioxidants, their effect on redox balance may vary in different environmental conditions. In particular, vitamin E can exert a prooxidant effect, especially under conditions of low radical flux, in a process called tocopherol-mediated peroxidation. Vitamin E exerts an antioxidant effect on LDL in the presence of a highly prooxidant environment; for lower levels of oxidative stress, vitamin E maintains its antioxidant activity only in the presence of physiological amounts of ascorbate, and, more interestingly, it becomes prooxidant in highly diluted plasma and in isolated LDL. Moreover, whereas vitamin E is effective against 1-electron oxidants (ie, radical oxidants), it is not able to neutralize oxidative damage caused by 2-electron oxidants (ie, nonradical oxidants), such as HOCl and ONOOH. Tyrosine is one of the amino acidic residues oxidized by ONOOH to form 3-nitrotyrosine. Thus, in the present study, the increased amount of nitrotyrosine we found in coronary arteries of normal pigs treated with antioxidant vitamins could be the result of this process.

N-nitration of eNOS has been demonstrated to impair its enzymatic activity, which, similar to S-nitrosylation, might be because of the reduced dimerization of its subunits. Hence, the decreased content of dimerized eNOS, found in the present study in V pigs, in conjunction with preserved total eNOS, likely reflected the nitration of the eNOS monomer, which prevents its coupling. This is supported by our observation of nitrotyrosine immunoreactivity in endothelial cells and by the higher nitrotyrosine immunoblotting after eNOS immunoprecipitation in V. The eNOS uncoupling, besides reducing NO production, is also known to increase reactive oxygen and nitrogen species production, possibly instigating a vicious cycle of oxidants generation.

Similar to vitamin E, high-dose supplementation with vitamin C is paradoxically associated with an increased free-radical-mediated DNA damage. Under normal condition, excess of toxic radicals of vitamin E and vitamin C in blood can be uptaken by various tissue recycling mechanisms, and oxidized-LDL particles that may form are rapidly cleared by the liver. In contrast, the same vitamin-derived radicals accumulate within the vessel wall and may not be as readily cleared, resulting in a prooxidative effect on the arterial structures. Supporting this phenomenon, a strong positive correlation was found between tissue vitamin E levels and tissue-lipid peroxidation in human atherosclerotic plaque.
Several studies have shown that long-term administration of antioxidant vitamins improved endothelium-dependent vasorelaxation in animal models in the presence of cardiovascular risk factors and increased oxidative stress.\textsuperscript{14,40,41} In the present study, chronic administration of vitamin C and vitamin E to normal pigs resulted in a significant impairment of the endothelium-dependent vasorelaxation in the coronary artery. Interestingly, it has been shown previously that whereas low doses of chronic \( \alpha \)-tocopherol supplementation improved endothelial function in high-cholesterol-fed rabbits, the use of higher doses worsened such a response, despite the greater apparent “protection” of LDL particles.\textsuperscript{40} This suggests that when antioxidant vitamin load exceeds the levels necessary to counteract endogenous oxidative stress, a shift from their protective antioxidant effect to a detrimental prooxidant effect can be seen. Our current study confirms this hypothesis by showing that antioxidant vitamin supplementation in normal animals, characterized by low basal oxidative stress, impairs the coronary endothelial function. Moreover, the presence of ameliorated plasma markers of LDL oxidation and unchanged TBARS values after vitamin supplementation highlights the dissociation between the redox status of the vessel wall and the plasma markers of LDL oxidation\textsuperscript{40} or a generic plasma marker of oxidative stress, such as TBARS. Indeed, in our study, enhanced oxidative stress was seen within the coronary arterial wall, demonstrated by an increase in nitrotyrosine and superoxide production.

We evaluated, for the first time, the effect of antioxidant vitamins on myocardial perfusion of large animals under normal conditions, showing no changes in basal perfusion but an important reduction in the response to cardiac stress. The reduced adaptation of myocardial perfusion to increased demand in V can be explained, at least in part, by the reduced vasoreactivity in epicardial arteries. To support this, previous studies from our group demonstrated that experimental conditions with coronary endothelial dysfunction were also characterized by impaired response of myocardial perfusion to adenosine and dobutamine, and improvement of endothelial function by antioxidant vitamin supplementation was able to restore myocardial perfusion.\textsuperscript{17,18} However, a microcirculatory dysfunction directly caused by vitamins and vitamin-derived radicals could also play a role in determining the abnormal myocardial perfusion response, and this is supported by the increased microvascular permeability after adenosine or dobutamine. This transient alteration in permeability can be considered a marker of microcirculatory vascular dysfunction and has been demonstrated previously in association with the presence of cardiovascular risk factors\textsuperscript{14,20} and correlated with tissue oxidative stress.

**Perspectives**

It may be speculated that the administration of high-dose antioxidant vitamins in healthy subjects, who presumably have a low oxidative stress level, could be detrimental. Therefore, specific studies in healthy human subjects are required, and antioxidants administration should be taken into consideration when dealing with patients with increased oxidative stress. In this regard, when evaluating the effect of antioxidants on clinical end points, including patients with low oxidative stress in the study population could result in an underestimation of the potential benefit of antioxidant vitamins to patients with high oxidative stress. This implies that the use of reliable markers of oxidative stress in vivo before commencement of antioxidant vitamin therapy should be considered.

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